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A method for generating antigen-presenting cells

Field of the Invention

The present invention relates to a method for the generation of antigen-presenting cells (APC), preferably bone marrow-derived dendritic cells (BMDC) or peripheral blood-derived dendritic cells (DC), as antigen carrier having immunostimulatory properties for anti-infective treatment and cancer vaccination comprising the steps of (a) exposing the APC to antigen and (b) treating the APC with a CpG oligonucleotide. Said APC are useful as an immune prophylactic or immune therapeutic agent against various cancerous and infectious diseases.

Background of the Invention

In the last century, major advances in vaccination and chemotherapy have accounted for the significant success in prevention and control of a variety of infectious diseases. At the beginning of the 21st century, however, infectious diseases remain to be the first cause of morbidity and mortality in developing countries and are still responsible for a significant proportion of public health problems in the developed world. For example, in 1998, the World Health Organization (WHO) reported that AIDS, tuberculosis, malaria and leishmaniasis caused more than 5 million deaths all over the world, and therefore they keep being a major group of human diseases to be targeted in the future.

Interestingly, these four diseases, although they are different in their origin from cancer, have common characteristics when it comes to a comparison of vaccination strategies that have been explored. In general, positive clinical results are missing in cancer vaccination and the various vaccination strategies have not yet yielded into a therapeutic modality of generally broad applicability producing regressions of metastatic lesions in individual patients. It is remarkable that independent of the goal of vaccination against cancer and infections, some features are in common: 1. no reliable effective vaccines are available, 2. chemotherapy is limited, 3. the causing agent is a transformed cell type or an intracellular pathogen, and 4. al-

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(87) Abstract: Described is a method for the generation of antigen-presenting cells (APC), preferably bone marrow-derived dendritic cells (BMDC) or peripheral blood-derived dendritic cells, as antigen carrier having immunostimulatory properties for anti-infective treatment comprising the steps of (a) exposing the APC with antigen and (b) treating the APC with a CpG oligonucleotide. Said APC are useful as an immune prophylactic or immune therapeutic agent against diseases like AIDS, tuberculosis, malaria or leishmaniasis.

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In the last years, laboratories have been particularly interested in the use of the most potent APC, the dendritic cells (DC), as "natural" adjuvants and potent inducers of a Th1 immune response. For this purpose, the model of murine leishmaniasis was used. It could be shown that after cutaneous infection with *Leishmania major*, only DC are able to migrate and transport the antigen from the skin to the lymph nodes, and are unique in providing the signals for initiation of the primary specific T-cell response. In addition, DC retain parasite antigen in an immunogenic form for prolonged periods, due to the increased stability of the MHC class II-peptide complexes, and may thus allow the sustained stimulation of parasite-specific T cells that maintain protective immunity against leishmaniasis. These observations prompted the scientists to explore the possibility to use DC as adjuvants for immunization against infectious diseases. These natural adjuvants for immunization against infectious diseases. These studies demonstrated that members of the DC family, epidermal Langerhans cells (LC), after ex vivo pulsing with *L. major* lysate, can induce long-lasting protection of otherwise susceptible BALB/c mice against subsequent challenges with virulent parasites. This protection was paralleled by a pronounced shift towards a Th1-like pattern. In contrast to the control animals in which a typical Th2 immune response was observed. Thus, one

6 can expect that DC can serve as an effective antigen delivery system for vaccination against infectious diseases and open the possibility for a potential use in therapy. This notion is supported by similar results obtained with other models of bacterial, parasitic, fungal and viral infections.

10 Availability of DC in sufficient numbers as needed in a therapeutic approach and quality to support clinical treatment of patients will in a long run decide about whether the innate therapeutic potential of DC can be used or not. If this problem is not solved the practical implications will be that DC will stay what they currently are, a powerful research tool.

15 DC constitute a rare but heterogeneous population phenotypically distinct from macrophages (DC are CD14⁺). DC are defined by their potency as APC and are distinct from other well known, but less potent, APC such as B cells and macrophages. DC have been shown to be derived from numerous lineages and dynamically shift their phenotype in response to the local inflammatory environment. The most powerful DC currently known and desirable for use in vaccination approaches are skin-derived DC sometimes referred to as "Langerhans cells" (LC). However, they constitute only 1-3% of the epidermal cells and their isolation from the skin is complicated. Blood DC represent a similarly small population as they contribute to less than 0.3% of the entire circulating blood-leukocyte population. The lack of adequate culture methods for DC is an additional limitation. Thus, in humans, sizable numbers of naturally occurring LC/DC are not accessible.

30 Other reported sources for DC are less differentiated cells like CD34⁺ progenitors derived from blood monocytes preparations or the uncommitted bone marrow-derived CD34⁺ cells. However, these cells have to be differentiated first *ex vivo* to acquire a DC phenotype.

35 Human monocyte-derived DC currently represents the easiest accessible source of DC. The number of monocytes available from blood is reasonable and the procedures involved are not too inconvenient for the donor. Generally, DC that have been used in vaccine protocols have been generated from monocyte preparations stimulated with IL-4 and GM-CSF or from monocyte derived precursors (CD34⁺ cells). In humans, monocytes derived cells incubated without IL-4 become activated macrophages. In the murine system, the use of IL-4 is not required to generate DC. In a first

5 step of preparation, peripheral blood mononuclear cells (PBMC) are isolated via density centrifugation. By this way, all red blood cells and the granulocytes are lost in one isolation step. Then the PBMC are cultured for 6 days in the presence of GM-CSF and IL-4. At day 6 the cells have lost CD14 (a monocyte lineage marker) and gained CD1a. Classical stimuli like lipopolysaccharide (LPS) can stimulate the (immature) DC to produce factors like IL-6, IL-8 and IL-12 (p40 and p70).

10 Despite of limitations posed by the inaccessibility of cutaneous DC they are the best-studied DC type. Much attention has been given to situations in which CD4⁺ and CD8⁺ T lymphocytes play a critical role and need to be activated. Cutaneous DC present tumor antigen as well as antigens from infectious agents in the context of class I molecules. Furthermore, LC are able to present exogenous antigens loaded onto class I molecules, a function unique to LC/DC and known as cross-priming. Thus DC can stimulate both T cells and B cells. This finding is of great importance because both the CD4⁺ and CD8⁺ T cells are a requirement for protective cancer immunity and need to be activated through class I-presented antigen. In addition to their unique antigen presentation function, cutaneous DC are equipped with extraordinary accessory functions: Together, these exquisite features enable DC to induce primary and secondary immune responses. For this reason, DC are often referred to as "nature's adjuvant" and this opens attractive options in the therapy of cancer and infectious diseases.

25 In immunotherapy of cancer, the role of cancer-specific CD4⁺ and CD8⁺ T cells for generating an antigen-specific and therapeutic immune response is undisputed and a prerequisite for concepts that foresee successful vaccination and are focused to prevention and control of cancer. Nevertheless, due to the lack of immunogenic tumor antigens, the absence of accessory signals and/or active immunosuppression, the natural or vaccination-induced immune response often fails and does not help to combat cancer. Experimental work generated from several laboratories indicates that cutaneous DC present tumor antigens in the context of class I molecules, which is a requirement for the activation of both CD4⁺ and CD8⁺ T cells to perform protective cancer immunity.

35 Dendritic cells (DCs) derived from monocytes have been used by few institutions in their current experimental immunotherapy protocols. The results of the studies are difficult to compare since the DC involved have not been

generated following a generally accepted standard and their phenotypes are different. The administration of the DC loaded with tumor-associated proteins or peptides resulted in the induction of immune responses against different types of malignant cells. Clinical responses such as stability of disease and tumor regressions have been reported in some patients, particularly with melanoma, myeloma, follicular non-Hodgkin's lymphoma and prostate cancer.

In the clinical trials with DC-based vaccines, a number of important limiting issues have become apparent. These include the optimal source and phenotype of DC, the type of antigen and method of loading DC with antigen, whether to induce differentiation/maturation of DC, the route and timing of immunization, and the appropriate clinical scenario.

The monocyte-derived DC currently used for cancer immunotherapy are not generated following a general standardized scheme. To further explore DC-based approaches, it is therefore very important to establish a protocol for the generation of DC in sufficient amounts and with potent immunostimulatory properties that are similar to those reported for LC/MHC class I-mediated antigen presentation, accessory functions, ability to induce a T helper response). The use of bone marrow precursor cells seems to be an alternative way to generate larger numbers of DC. However, the same questions arise: what stimulation conditions are required to differentiate them to become the ideal antigen carrier? With respect to shifting and modulating immune responses certain products of bacteria and helminths stimulate APC and as well DC to prime and activate preferentially Th1 or Th2 cells, respectively. Among the preferred bacterial products are the oligonucleotides that have been shown to be immunostimulators of B cells, NK cells, peripheral blood mononuclear cells (PBMC) and blood dendritic cells (see US6429189, US 6207846). In various studies it has been shown by Krieg et al. that an unmethylated cytosine-guanine (CpG) di-nucleotide motif is central for the immunostimulatory property and represented by the general formula:

5' X₁CG X₂3', wherein X₁ is selected from the group consisting of A, G and T; and X₂ is C or T. CpG containing nucleotides have been reported to be in range of 8 to 40 base pairs. However, nucleic acids of any size are immunostimulatory if sufficient immunostimulatory motifs are present

The authors (Krieg et al.) demonstrate that CpG activates PBMC and that within the various cells types present in monocyte preparations the CpG DNA directly activates the macrophages, which respond with a release of various cytokines (IL-6, GM-CSF and TNF-alpha). Both B cells and NK cells have been shown to be specifically activated by ODN 1668. Krieg et al. furthermore demonstrate that in contrast to monocyte derived dendritic cells it is only the low numbered (0.2%), naturally occurring blood dendritic cells that is susceptible to CpG stimulation. Krieg et al. about white monocyte-derived dendritic cells in US6429189: "DC can be obtained in large numbers,however upon withdrawal of IL-4 loss their DC character-istics,.....IL-4 induces Th2 immune response which may not be optimal a specific cytotoxic T cell response,.....We found that monocyte-derived dendritic cells are sensitive to LPS but surprisingly are not activated by CpG motifs. It is believed that the inability of monocyte-derived DC to respond to CpG might be due to the unphysiologic methods by which these cells are prepared." Throughout their work Krieg et al. have profiled the natural occurring DC of the peripheral blood as the prime target of CpG action. However as it has been pointed out before, these physiological DC are because of their limited number not a preferred cell type when it comes to large scale use of therapeutic cells in mammals.

Thus, the technical problem underlying the present invention is to provide in a large scale APC, preferably DC, which can serve as antigen carriers, or natural adjuvant, for anti-cancer and anti-infective treatments.

5

Summary of the invention

The solution to the above technical problem has been achieved by providing the embodiments characterized by the claims and as follows: It could be demonstrated that particular DC, i.e., BMDC or monocyte-derived DC, can be manipulated in vitro with specific maturation stimuli, i.e., CpG oligonucleotides, resulting in the generation of activated BMDC that exhibit a striking capacity to induce a T cell immune response and protect mammals against an otherwise lethal infection with intracellular pathogens and from cancer.

DC were generated from bone marrow progenitors as described by Lutz et al. and the resulting cell population had a typical DC morphology with a myeloid DC phenotype (MHC class II⁺, CD80⁺, CD86⁺, CD40⁺, ICAM-1⁺, CD11c⁺), and potent MHC class I dependent antigen-presenting functions in allogeneic MLR and in a proliferation assay with Leishmania-specific T hybridoma cells. After 10 days of BMDC culture, the non-adherent cells were collected, resuspended at in culture medium containing GM-CSF and pulsed with antigen.

As a model system, experimental leishmaniasis with *Leishmania* major was used. A single vaccination of mice for example with DC which had been pulsed in vitro with *Leishmania* antigen and treated with a CpG oligonucleotide for maturation (DC/CpG/*LeishAg*) mediated complete protection against subsequent infection with the parasite *Leishmania*. Control mice which obtained *Leishmania* antigen or the CpG oligonucleotide alone were not protected. Analysis of the underlying immunological mechanism revealed that vaccination with DC/CpG/*LeishAg* induced a cell-mediated immune response of the protective type, i.e., an immune response mediated by CD4⁺ type 1 T helper cells (Th1). The protective effect was stable and long-lasting, i.e., more than 20 weeks after secondary challenge the mice did not exhibit any signs of disease. Using this approach for vaccination against infection or cancer, it can be expected that DC generated from humans or other animals will induce a protective immune response in the treated individual.

Brief description of the drawings

Figure 1: Lesion development in BAL B/c mice vaccinated with BMDC preparations and infected one week later with *L. major* parasites

BMDCs were produced and incubated with the different treatments as described in Material and Methods. A and B represent two independent experiments and show the average in footpad swelling for every group \pm SEM (n=5).

Figure 2: Clinical cure of murine cutaneous leishmaniasis induced by CpG-matured lysate-pulsed BMDC is associated with a significant reduction in parasite burden

Control non-vaccinated mice and protected mice from experiment shown in Fig. 1B were sacrificed and the amount of viable parasites was determined by a limiting dilution procedure (A). Samples from footpad suspensions were smeared, stained with Giemsa and observed with light microscopy (B). Pictures displayed are representative from each group.

Figure 3: The pattern of cytokine expression by lymph node cells from protected mice indicates a shift towards a Th1-like immune response

Pooled lymph node cell suspensions were prepared from some relevant groups shown in Fig. 1B and were incubated for 72 hours in the absence (open) or presence (filled) of *Leishmania* antigen. Supernatants were assayed for the production of IL-2 (A), IFN- γ (B) and IL-4 (C) by ELISA.

Figure 4: The production of *Leishmania*-specific IgG antibodies in protected mice correlates with a Th1 immune response

Sera from individual mice belonging to the experimental groups shown in Fig. 3 were analyzed for the presence of total IgG (A), IgG1 (B) and IgG2a (C) anti-*Leishmania* antibodies by ELISA. Results are shown as O.D. and

5 the average is indicated with the bar. The ratio of IgG2a/IgG1 was calculated for each mouse and is shown in D.

10 **Figure 5: Protection against murine cutaneous leishmaniasis by CpG-matured lysate-pulsed BMDC can be also shown in resistant C57BL/6 mice**
 BMDC were treated and i.v. injected into mice one week before parasite challenge as described in material and methods. Footpad swelling was then weekly registered (A) and the parasitic load in pooled footpads was qualified after 8 weeks of infection (B).

15 **Figure 6: (A) Treatment with CpG-matured lysate-pulsed BMDC mediates solid protection against re-infection**

Cured mice from the experiment shown in Fig. 1A were re-challenged with 5×10^6 infective parasites and the lesion development was followed up weekly.

20 (B) Evaluation of the therapeutic potential of CpG-matured lysate-pulsed BMDC

Mice were infected, i.v. injected with CpG-matured lysate-pulsed BMDC at the time-points indicated in the top of the figure and footpad swelling monitored.

Figure 7: IL-12 expression by BMDC used for vaccination

BMDC were generated, treated as indicated for 36 hrs and supernatants were separated from cells by centrifugation. Cells were used to amplify the mRNA for IL-12 p40 and IL-12 p35 subunits by RT-PCR, as described in Material and Methods (A). Supernatants were assayed for IL-12 p70 expression by ELISA (B).

5 **Detailed description of the invention**

In one aspect, the present invention relates to a method for the generation of an APC as antigen carrier having immunostimulatory properties for anti-infective and anti-cancer treatment comprising the following steps:

(a) exposing the APC to antigen (e.g. by pulsing APC with antigen); and

(b) treating the APC with a CpG oligonucleotide.

APC suitable for the method of the present invention comprise different subsets of the DC family with BMDC or peripheral blood-derived DC being preferred. Methods for the generation of DC and the separation of said cells from non-APC are known to the person skilled in the art and described, e.g., in Lutz et al., J. Immunol. Meth. 223: 77-92 (1999); Romani et al., J. Immunol. Meth. 196: 137-151 (1996); Thurner et al., J. Immunol. Meth. 223: 1-15 (1999). Methods for pulsing of the APC in general or specific DC with the antigen are also known to the person skilled in the art and described, e.g., in Flohé et al., Eur. J. Immunol. 28: 3800-3811 (1998) as well as in Example 1(D), below.

The person skilled in the art knows how to carry out treatment of the APC with a CpG oligonucleotide, e.g., by following the instructions given in Example 1(D), below. Steps (a) and (b) can be carried out separately or simultaneously.

Preparation of the CpG oligonucleotide can be carried out according to conventional methods (cf. Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory Press, NY, USA).

The term "having immunostimulatory properties" comprises the capability of the matured APC to provide a protective immune response.

Infectious disease related antigens according to this invention is whole cell lysate and antigen mixtures derived from mycobacteria, chlamydia, influenza virus, HPV, HBV, HCV, EBV origin, and molecular defined antigens such as Leif, elongation factor 4 and LACK from Leishmania, listeriolysin from *Listeria monocytogenes* and *Toxoplasma gondii* antigens such as for instance SAG1 and SAG2.

The human cancer antigens recognized by CD8⁺ T cells are selected from the group of cancer-*laetis* antigens (e.g. MAGE-3, BAGE, GAGE, NY-ESO-1),

5 melanocyte differentiation antigens (e.g. Melan-A/Mart-1, tyrosinase, gp100), overexpressed antigens (e.g. Her2/neu, erbB1, p53, MUC-1) and point mutated antigens (e.g. beta-Catenin, MUM-1, CDK-4, p53, ras).

10 In a preferred embodiment of the present invention a not naturally occurring dendritic cell (DC) having specific antigen presentation properties in a mammal comprising a specific disease related antigen and a CpG molecule is generated and used. Said DC derives from CD34+ bone marrow cells precursor cells or peripheral blood monocytes and the APC are BMDC or peripheral blood-monocyte derived DC as antigen carrier having immunostimulatory properties for anti-infective and cancer treatment

15 treatment and the method comprises the following steps:

(a) obtaining bone marrow cells from femurs and/or tibiae or isolating DC precursor cell from peripheral blood monocyte preparations;

20 (b) culturing the cells under conditions allowing to generate DC;

(c) pulsing the isolated DC with antigen; and

(d) treating the DC with a CpG oligonucleotide.

25 Methods of the steps (a) and (b) are commonly known and, moreover, described in Example 1(D), below. Steps (c) and (d) are preferably performed simultaneously. If performed sequentially, step (d) is performed before (c).

30 In a more preferred embodiment of the method of the present invention, the CpG oligonucleotide comprises the nucleic acid sequence 5'-TTCATGACGTTCCCTGATGCT-3'. However nucleic acids represented by the general formula(5' X1 CG X2 3') may be used, wherein X1 is selected from the group consisting of A, G and T; and X2 is C or T as well. A reasonable length for CpG containing nucleotides has been reported to be in range of 8 to 40 base pairs.

35 The immunogenicity of the antigens used in this invention may be substantially increased by including adjuvants. A preferred embodiment of a vaccine based on the present invention therefore contains QS21, incomplete

5 Freund's adjuvants, IL-2, IL-12, GM-CSF, MPL or an AGP such as RC-528.

In a further more preferred embodiment of the method of the present invention the APC, preferably BMDC, are characterized by their ability to induce a T-helper immune response. This ability can be assayed by standard assays, e.g., the assay described in Example 5, below.

10 The term "antigen" as used herein comprises a lysate of a pathogenic microorganism, e.g., parasite, (see, for preparation, e.g. Example 1(D), below) or one or more purified proteins of the pathogenic organism. Preferably, the antigen is an isolated protein, or a mixture of such proteins of a microorganism and/or the microorganism is an intracellular pathogen. It is especially preferred that the microbial antigen is selected from the group consisting of (1) cells or an extract, (2) an isolated microbial antigen, (3) an isolated nucleic acid representing the antigen operable linked to a promoter for expressing the isolated antigen, or functional variant thereof, (4) a host cell expressing the isolated polypeptide or a functional variant thereof.

20 The method of the present invention is useful for providing immune protection against a variety of microorganism, preferably intracellular pathogens (parasites), e.g., HIV, Mycobacterium tuberculosis, Plasmodium, Leishmania, Salmonella, Listeria, Toxoplasma and Chlamydia.

25 The present invention also relates to APC having immunostimulatory properties, preferably BMDC or peripheral blood-derived DC, which are obtainable by the methods of the present invention described above and exemplified in the Examples, below, as well as a pharmaceutical composition containing said cells, preferably in combination with suitable pharmaceutical carriers. Examples of suitable pharmaceutical carriers are well known in the art and comprise buffered aqueous solutions. Such carriers can be formulated by conventional methods and can be administered to the subject at a suitable dose. Administration of the suitable compositions for vaccination may be effected by different ways, e.g. by intravenous, intraperitoneal, subcutaneous, intramuscular or intradermal administration. The route of administration, of course, depends on the nature of the disease, e.g. kind of pathogen or parasite, and the kind of APC contained in

the pharmaceutical composition. The dosage regimen will be determined by the attending physician and other clinical factors. As is well known in the medical arts, dosages for any one patient depends on many factors, including the patient's size, body surface area, age, sex, the particular APC to be administered, time and route of administration, the kind of pathogen, general health and other drugs being administered concurrently. DC pulsed with antigen and treated with CpG and/or pharmaceutical compositions of the present invention may be used as a vaccine. Accordingly, in a further aspect, the present invention relates to a method for inducing an immunological response in a mammal that comprises inoculating the mammal with DC pulsed with antigen and treated with CpG and/or pharmaceutical compositions of the present invention, adequate to produce an antibody and/or T cell immune response, including, for example, cytokine-producing T cells or cytotoxic T cells, to protect said animal from disease, whether that disease is already established within the individual or not. An immunological response in a mammal may also be induced by a method comprising delivering the antigen of the present invention via a vector directing expression of the polynucleotide and coding for the polypeptide *in vivo* in order to induce such an immunological response to produce cytotoxic and memory T cell or antibody to protect said animal from diseases of the invention. One way of administering the vector is by accelerating it into the desired cells as a coating on particles or otherwise. Such nucleic acid vector may comprise DNA, RNA, a modified nucleic acid, or a DNA/RNA hybrid. For use as a vaccine, the DC pulsed with antigen and treated with CpG are normally provided as a vaccine formulation (composition). The formulation may further comprise a suitable carrier. A preferred route for administration is parenterally (for instance, subcutaneous, intra-muscular, intravenous, or intra-dermal injection). Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions that may contain anti-oxidants, buffers, bacteriostatics and solutes that render the formulation isotonic with the blood of the recipient, and aqueous and non-aqueous sterile suspensions that may include suspending agents or thickening agents. The packages formulations may be presented in unit-dose or multi-dose containers, for example, sealed ampoules and vials and may be stored in a freeze-dried condition requiring only the addition of the sterile liquid carrier immediately prior

to use. The vaccine formulation may also include adjuvant systems for enhancing the immunogenicity of the formulation, such as oil-in water emulsions and other systems known in the art. Another way to enhance immunity may require including cytokines and growth factors such as IL-2, IL-4, IL-12, alpha-IFN, GM-CSF. The dosage will depend on the specific activity of the vaccine and body weight of the recipient and can be readily determined by routine experimentation.

Finally, the present invention relates to use of a APC as described above, preferably a BMDC or peripheral blood-derived DC for the preparation of a pharmaceutical composition, preferably an immune prophylactic composition or immune therapeutic composition, for the treatment of a disease caused by an intracellular pathogen. Preferred diseases are AIDS, tuberculosis, malaria, salmonellosis, listeriosis, toxoplasmosis or leishmaniasis.

The following examples explain the invention in more detail.

Example 1: General methods

(A) Mice. Female BALB/c and C57BL/6 mice were purchased from Charles River Breeding Laboratories (Sulzfeld, Germany). Animals were 6 to 8 weeks old at the onset of experiments and were kept under conventional conditions.

(B) Parasites and preparation of antigen. Parasites of the *Leishmania* major isolate MHOM/IL/81/FE/BNi (Solbach et al., Infect. Immun. 54: 909 (1986)) were maintained by passage in BALB/c mice and were grown in conventional blood agar plates *in vitro*. For the preparation of total *L. major* lysate, stationary-phase promastigotes were collected, washed three times, resuspended at 1×10^9 /ml in PBS and subjected to three cycles of freezing and thawing.

(C) Oligonucleotides. The oligonucleotide 1688 (CpG ODN, 5' TCCAT-GAGCTTCCTGATGCT 3') and the control AT-rich oligonucleotide (non-CpG ODN, 5' ATTATTATTATTATTA TTAT 3') were synthesized by MWG (Ebersberg, Germany) and were not phosphorothioate-modified.

(D) Preparation and culture of bone marrow-derived dendritic cells (BMDC). Dendritic cells (DC) were generated from bone marrow progenitors using the protocol of Lutz et al., J. Immunol. Meth. 223: 77-92 (1999)

5 with minor modifications. Briefly, total bone marrow cells were obtained from femurs and tibiae after flushing with a syringe containing PBS. Cell suspension was washed and resuspended in culture medium (Click RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 10 mM Hepes buffer, 80 µg/ml penicillin, 20 µg/ml gentamycin, 17 mM NaHCO₃ and 0.05 mM 2-mercaptoethanol). At day zero, 2×10^6 cells were seeded in bacteriological petri dishes in a total volume of 10 ml culture medium containing 200 U/ml recombinant murine granulocyte/macrophage colony-stimulating factor (GM-CSF; Peprotech/Tebu, Frankfurt, Germany). Additional 5 ml of culture medium containing 200 U/ml GM-CSF were added at days 3 and 8. After 10 days of culture, non-adherent DC were collected, resuspended at 1×10^6 cells/ml in fresh culture medium containing 200 U/ml GM-CSF and were incubated overnight (approx. 18 hr) with 30 µl of parasite lysate per ml of culture volume (approx. equivalent to 30 parasites per cell) for antigen pulsing. For some experimental groups, concomitant treatment with recognized inducers of BMDC maturation (lipopolysaccharide, LPS: 1 µg/ml, Sigma, Heidelberg, Germany); CpG and non-CpG ODNs: 25 µg/ml; anti CD40 mAb: 5 µg/ml, Pharmingen, Hamburg; and tumor necrosis factor alpha, TNF-α: 500 U/ml, Peprotech/Tebu, Frankfurt, Germany) was included during pulsing. Control groups with only CpG, non-CpG ODNs and LPS were also included. After overnight incubation, the cells were washed to remove soluble parasite antigen and maturation inducers, and resuspended in PBS for further use.

(E) *Treatment of mice:* After antigen pulsing/maturation, BMDC were washed and resuspended in PBS, and 5×10^6 cells were injected intravenously (i.v.) into the tail vein of naive mice. Control mice were injected with 2×10^6 (BALB/c mice) or 2×10^6 (C57BL/6 mice) stationary-phase *L. major* promastigotes into the right hind footpad. The course of infection was monitored weekly by measuring the increase in footpad size, compared with the uninfected contralateral footpad. For re-infection experiments, mice were infected with 5×10^6 parasites into the left hind footpad 9 weeks after the primary infection, which means 3 weeks after complete healing of primary infection. For therapeutic immunization, mice were initially infected and subsequently treated on days 7, 0 + 7, 7 + 14 or 14 + 21 post-infection by i.v. injection with 5×10^6 BMDCs.

(F) *Determination of the parasite load.* In order to analyze whether effective leishmanicidal mechanisms were taking place at the site of infection, the amount of viable parasites in the footpads was determined by a limiting dilution technique. Briefly, after 5-8 weeks post infection the right foot was removed, washed with ethanol and rinsed three times with PBS. Preparation of soft tissues was performed by making some elts with a sterile scalpel and by macerating the foot in a cell strainer. Cell suspensions were then passed through a 30G needle in order to assure the release of intracellular parasites. Subsequently, suspensions were centrifuged for 5 minutes at 100g in order to separate tissue clumps and debris. Serial dilutions of the supernatant in 100 µl/well were seeded into 96-well microculture blood-agar plates. For each dilution, replicates of 20 wells were set up. After 10 days of incubation at 28°C in a humidified atmosphere with 5% CO₂, the cultures were scored for the presence of parasites using an inverted microscope. The estimation of the number of parasites per footpad was done by multiplying the reciprocal of the last dilution showing at least one positive well with the initial dilution factor. For some experimental groups, 10 µl of the footpad cell suspension were smeared onto a glass slide, stained with Giemsa and observed in a conventional light microscope for the presence of *L. major* amastigotes.

(G) *Measurement of cytokine production.* Lymph nodes draining the infected footpads were removed 5 weeks after infection. After preparation of single-cell suspensions, 1×10^6 cells were cultured in 1 ml volume (24-well plates) in the absence or presence of 10 µl of parasite lysate for 72 hours. Thereafter, culture supernatants were harvested for the determination of the cytokines IL-2, IL-4 and IFN-γ by sandwich ELISA, as published previously (Flohé et al., Eur. J. Immunol. 28:3800-3811 (1998)). IL-12p70 was also measured by sandwich ELISA in supernatants of BMDC cultures after 20 hours of pulsing/maturation.

(H) *Determination of Leishmania-specific IgG antibodies.* Mice of the experiment shown in Fig. 1B were sacrificed 5 weeks after infection and *Leishmania*-specific IgG, IgG1 and IgG2a serum levels were assayed by ELISA. Plates were coated with total lysate (equivalent to 5×10^6 parasites/well) and incubated overnight with mouse serum (dilutions: 1:100 for total IgG; 1:50 for IgG1 and IgG2a). For total IgG a second antibody (anti-mouse IgG-alkaline phosphatase conjugate) was incubated for 1 hour and

5 developed with a chromogenic phosphatase substrate. For IgG1 and IgG2a, 1-hour incubation with an isotype-specific second antibody (biotinylated rabbit anti-mouse IgG1 and IgG2a, respectively), 1 hour with streptavidin-conjugated alkaline phosphatase and final substrate development were used. Relative levels of antibodies are presented in optical density (O.D.).

10 (7) RT-PCR. Total RNA was isolated from BMDc cultures after 36 hours of different pulsing/maturation treatments, using the RNeasy total RNA extraction kit (Qiagen, Hilden, Germany) and 2 µg of RNA were reverse transcribed (Qiagen, Hilden, Germany). Primers for IL-12 p35, IL-12 p40 and β-actin (MWG Biotech, Ebersberg, Germany) were used in a PCR reaction to estimate the relative amount of their respective mRNAs.

20 Example 2: CpG-matured / yeast-pulsed BMDc protect BALB/c mice from cutaneous leishmaniasis

Recently, it has been reported that Langerhans cells that had been pulsed with *Leishmania* antigen confer protection against murine leishmaniasis. Initial attempts to reproduce this protective effect with a different population of DC, the BMDc, were unsuccessful. Several modifications of the protocol with regard to the time of BMDc generation, the amount of BMDc injected into the mice and different conditions of antigen pulsing were performed. However, no protection against infection could be observed (not shown). Thus, additional maturation stimuli of BMDc seemed to be required. Therefore, a series of experiments was performed in which cells were not only pulsed with parasite yeast (as the source of antigen), but in addition treated with inducers of BMDc maturation, including LPS, anti-CD40 antibodies, CpG ODN and TNF-α. Two independent and representative experiments are shown in Fig. 1. Again, antigen-pulsed BMDc were not able to induce protection against leishmaniasis (Fig. 1B). When antigen-pulsed BMDc were additionally treated with the maturation inducers LPS, anti-CD40 and TNF-α, or combinations of those stimuli, BMDc were also unable to protect against leishmaniasis (Fig. 1A and 1B). In contrast, immunization of mice with antigen-pulsed BMDc that had been cultured in the presence of CpG ODN conferred complete protection

5 against subsequent infection with *L. major* in otherwise susceptible BALB/c mice (Fig. 1A and 1B). All mice that had been vaccinated with those cells developed only a minor footpad swelling (always less than 1 mm, Fig. 1A and 1B), which peaked 3 weeks after infection, and were completely cured after 6 weeks (Fig. 1A). None of the mice in this group showed any sign of ulceration. The course of lesion development in control groups immunized with non-pulsed CpG-treated BMDc or pulsed BMDc treated with a non-CpG motif AT-rich ODN was comparable to the PBS control group (Fig. 1B). These findings demonstrate that a single i.v. injection with antigen-pulsed CpG-matured BMDc mediates complete protection against murine leishmaniasis.

20 Example 3: Clinical cure correlates with a significant reduction in parasite burden

It was analyzed whether the protection induced by CpG-matured antigen-pulsed BMDc is paralleled by an effective control of parasite replication at the site of infection. Fig. 2A shows the parasite loads in individually analyzed mice from the protected and the control groups. All mice that had been vaccinated with CpG/antigen-BMDc had a significantly lower parasite burden than the control mice. On average, there was a more than 10⁴ fold reduction in the number of parasites per footpad (7.3 x 10⁴ and 1.2 x 10⁴ for control and protected groups, respectively). When smears from the control footpads were analyzed under the microscope, an uncountable high amount of parasites was seen and, as shown in Fig. 2B, macrophages were typically full of intracellular parasites, indicating active replication. In contrast, in samples obtained from the protected footpads, parasites could hardly be detected and the typical observation was the presence of no or very few intracellular amastigotes (Fig. 2B). These results indicate that the protection induced by immunization with CpG-matured antigen-pulsed BMDc in susceptible mice is due to an acquired ability to efficiently activate anti-*Leishmania* effector mechanisms.

6 Example 4: CpG-matured / lysate-pulsed BMDC induce a shift in the cytokine profile

In order to determine whether the protection induced by BMDC is associated with a different profile in cytokine expression, the secretion of IL-2, IFN- γ and IL-4 by lymph node cells was assessed. Mice from the most relevant experimental groups shown in Fig. 1B were sacrificed 5 weeks after infection, and total lymph node cells were cultured for cytokine analysis by ELISA. The levels of IL-2 in the absence of *Leishmania* antigen ranged between 7.6 and 20.7 ng/ml with the maximal level exhibited by the protected group that had been vaccinated with BMDC-lysate-CpG. However, this difference was strikingly enhanced when *Leishmania* antigen was added to the culture. A 13-fold higher level of this cytokine was observed in the protected compared with the control and 2 to 4-fold higher than the other groups (Fig. 3A). An even more pronounced difference was observed when IFN- γ levels were determined. As shown in Fig. 3B, a 10-fold increase was observed in the absence of antigen when the protected group is compared with the control group and 2 to 7-fold when compared with other groups. When *Leishmania* antigen was present in the cultures, a 151 and 18 to 60-fold higher level of IFN- γ was observed when protected group is compared with control and the other groups respectively (Fig. 3B). In contrast to IL-2 and IFN- γ , lymph node cells from mice belonging to the protected BMDC-lysate-CpG group secreted no detectable, or very low, levels of IL-4 in the absence or presence of antigen, respectively (Fig. 3C). Some of the non-protected groups were also low IL-4 producers. Thus, in mice treated with CpG-matured / lysate-pulsed BMDC, the cytokine profile induced in lymph node cells was strongly shifted towards Th1-like immune response.

35 Example 5: The pattern of *Leishmania*-specific IgG antibodies correlates with the induction of a Th1 immune response in CpG-matured/lysate-pulsed BMDC- vaccinated mice

It is well known that different IgG subclass profiles correlate with Th1 or Th2 immune response. The presence of high levels of IgG1 and low tiers

of IgG2a anti-*Leishmania* antibodies is associated with a Th2 response and the reverse distribution with a Th1 response. Thus, it was investigated whether the pattern of IgG subclass production was shifted towards the Th1-type response in the protected group. Mice from the most relevant experimental groups showed in Fig. 1B were sacrificed 5 weeks post infection, and the relative levels of total IgG, IgG1 and IgG2a antibodies were determined by ELISA. As shown in Fig. 4A, the levels of *Leishmania*-specific total IgG antibodies were variable but significant in all experimental groups. When the IgG subclass distribution was determined, a clear tendency to produce low IgG1 and high IgG2a levels was observed in the serum of protected mice that had been treated with BMDC-lysate-CpG (Fig. 4B and 4C). Some groups showed low levels of IgG1 and some high levels of IgG2a, but only the protective CpG matured/lysate-pulsed BMDC were able to induce the combination of both. A simpler parameter to see Th1-like shifting seems to be the relative ratio of IgG2a to IgG1, with higher values indicating Th1 induction. As shown in Fig. 4D, the protected BMDC-lysate-CpG group exhibited the highest IgG2a/IgG1 average ratio which was 4 times higher than for the control group (1.4882 and 0.3661, respectively). Some other groups showed higher ratio values than the control group due to higher IgG2a levels, but in contrast to the protected group, they also exhibited higher levels in IgG1 than the control group. Taken together, these results indicate that only the protected group of mice, which was vaccinated with CpG-matured lysate-pulsed BMDC, produces a pattern of anti-*Leishmania* antibodies that correlates with the induction of a strong Th1 immune response after infection with virulent *L. major*.

Example 6: The protective effect of CpG-matured/lysate-pulsed BMDC is also observed in the resistant strain of C57BL/6 mice

It was investigated whether this approach is also applicable to a different strain of mice which is resistant to *L. major* infection. As very well known (and shown in Fig. 5A, control group), C57BL/6 mice develop a limited inflammation in the footpad after infection and finally cure after 6-8 weeks of infection. However, when these mice are vaccinated with CpG-

matured/lysate-pulsed BMDC one week before the infection, a significant reduction in the footpad swelling is observed, with a lower maximal peak and faster healing (Fig. 5A). When these mice are vaccinated with BMDC alone, an initial unspecific effect is observed. However, these mice reached a footpad swelling comparable to the control after 4-5 weeks post-infection (Fig 5A). As expected, vaccination with BMDC treated with CpG infection showed no effect. In contrast to BALB/c mice, C57BL/6 mice vaccinated with BMDC pulsed with lysate in the absence of CpG treatment also showed a reduction in lesion development, when compared with non-vaccinated mice, but this effect was less pronounced than that induced by antigen-pulsed BMDC further matured by CpG ODN treatment (Fig. 5A). When the parasite load of the different vaccination groups was analyzed, a striking correlation with the clinical outcome was observed. The parasite numbers of mice with non-protective treatment were similar to those of the control (Fig 5B). Mice vaccinated with lysate-pulsed BMDC showed a 10-fold reduction in the parasite load. Most notably, those mice vaccinated with CpG-matured lysate-pulsed BMDC had approximately 100-fold less parasites in the footpads (Fig. 5B). These results demonstrate that vaccination with CpG-matured lysate-pulsed BMDC induces a significant protective effect, with a reduction of the parasite load at the site of infection, in both BALB/c and C57BL/6 mice.

Example 7: Resistance induced by CpG-matured / lysate-pulsed BMDC immunization is solid and protects against re-infection

Because of the exceptional efficacy of CpG-matured/lysate-pulsed BMDC in mediating protection (total cure of 100% of the mice, Fig. 1), it was investigated whether the mice that resolved the primary infection were able to resist a second challenge with parasites. To this end, the 10 mice that completely cured in the experiment shown in Fig. 1A were rechallenged with 0.5×10^6 metacyclic parasites (2.5-fold more than the primary infectious dose) 10 weeks after the first challenge. The results in Fig. 8A show that solid immunity was established by immunization with these BMDC, since the swellings developed after secondary infection was even lower than those after primary challenge. Rechallenged mice showed an almost

unreadable footpad swelling (less than 0.5 mm) and most of them completely cured after 3 weeks after the secondary infection. This group of mice was followed up for more than 20 weeks after secondary challenge without any sign of disease.

Example 8: The immunotherapeutic effect of CpG-matured/lysate-pulsed BMDC is dependent on the time of BMDC administration

Given the unusual potency of these cells in inducing a long-lasting protective Th1 immune response, the next question to address was whether it is possible to cure an already established *Leishmania* infection. For this purpose, a series of experiments was designed in which naive mice were infected and subsequently treated with CpG-matured/lysate-pulsed BMDC at different time points. The results shown in Fig. 6B indicate that under our experimental conditions, in a very limited time window of not longer than 7 days after infection, it is still possible to redirect the immune response towards a protective phenotype. When mice are treated on day 0 (1 hour post infection) and one week after infection, a very clear therapeutic effect is observed. When therapy is performed one and two weeks after infection the effect is reverted, since not only no curative effect is observed but also the treatment seems to be exacerbating. A similar curve of disease progression is observed when a single therapeutic dose is injected one week after infection, indicating that the therapeutic efficacy exerted by the schedule 0+7 days p.i. is more dependent on the first dose than the second, and strongly challenges the use of this approach at least in this model and under our experimental conditions. Finally, two therapeutic doses on days 14 and 21 p.i. did not show any effect as evidenced by a kinetic in disease progression comparable to that from the control group (Fig. 6B). Thus, a therapeutic application of BMDC in murine leishmaniasis is possible. However, the time of administration seems to be critical for efficacy.

5

Example 8: CpG-matured / lysate-pulsed BMDC express IL-12

To explore the mechanisms involved in the activation of the protective Th1-like immune response observed in mice vaccinated with CpG-matured/lysate pulsed BMDC, the expression of IL-12 was analyzed. This cytokine is formed by the subunits p40 and p35 and is known to play a key role in the development of Th1 cells. For this purpose, BMDC were treated as described in Example 1, and after 36 hours total RNA was isolated and levels of IL-12 p35 and p40 mRNAs were determined by RT-PCR. Supernatants of the same cultures were also collected and the active p70 form of the protein was measured by ELISA. As shown in Fig. 7A, p40 and p35 mRNA were differentially regulated by pulsing/maturation stimuli in BMDC. CpG and non-CpG ODN as well as LPS induce a very strong up-regulation of p35 mRNA while parasite antigen pulsing down-regulates both basal and induced expression. Among the groups having been treated by pulsing and maturation stimuli, BMDC-CpG combination showed the maximal p35 mRNA level. In contrast to p35, basal levels of the p40 mRNA were apparently unchanged by pulsing only. However, except for lysate-CpG combination, pulsing down-regulated the inducible expression (see LPS and CpGco alone versus LPS-Lys and CpGco-Lys, respectively). Again, among groups having been treated by pulsing and maturation stimuli, BMDC-CpG combination showed the maximal p40 mRNA level. Active p70 protein levels in supernatants were also dependent on the pulsing/maturation treatment as shown in Fig. 7B. As expected, maximal levels of p70 subunit were induced by CpG ODN treatment of BMDC. No p70 IL-12 was detectable in non-treated, pulsed only, and CD40 or TNF-alpha-matured pulsed cultures. However, in spite that again pulsing down-regulated the LPS- and CpG-induced p70 production, once more the last one was the treatment exhibiting the maximal level of functionally active p70 IL-12 among pulsed/matured groups (at least doubling amounts). All these results suggest that the protective effect observed when susceptible mice are vaccinated with BMDC pulsed with *Leishmania* lysate and matured with CpG ODN, is due to the induction of a strong Th1 immune response, which is correlated with the ability of these cells to secrete active p70 IL-12.

5

Claims

1. A not naturally occurring dendritic cell (DC) having specific antigen presentation properties in an individual comprising a specific disease related antigen and a CpG molecule, wherein said DC derives from CD34⁺ bone marrow precursor cells or peripheral blood monocyte preparations.
2. A DC according to claim 1, wherein the CpG oligonucleotide comprises the nucleic acid sequence
5'-TTCATGACGTTCTCTGATGCT-3'
3. A DC according to claims 1-2, wherein said DC was obtained by prolonged exposure to IL-4 and GM-CSF in vitro.
4. A DC according to claims 1-3, wherein the antigen is a microbial or a cancer antigen.
5. A DC according to claims 4, wherein the microbial antigen derives from a parasite.
6. A DC according to claims 5, wherein the parasite is *Leishmania*.
7. A DC according to claims 4, wherein the specific antigen presentation property comprises a Th1 type immune stimulatory response.
8. A pharmaceutical composition comprising a DC of any of the claims 1-7, optionally together with a pharmaceutically acceptable carrier, diluent and excipient.

Fig. 2

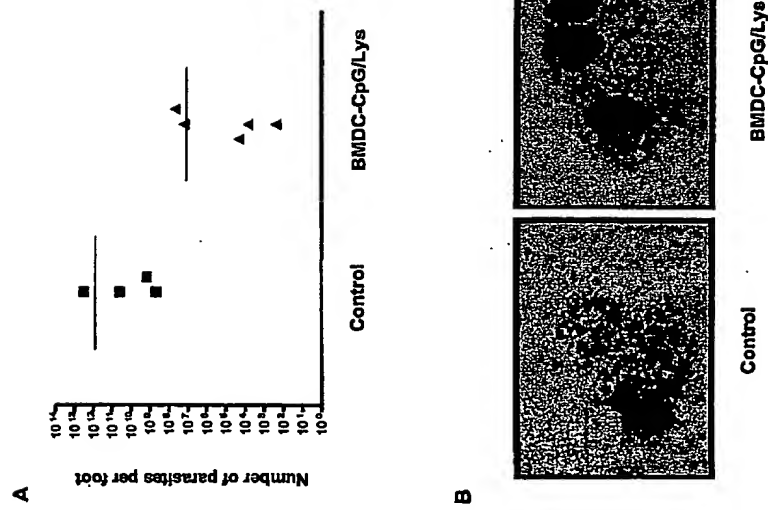


Fig. 3

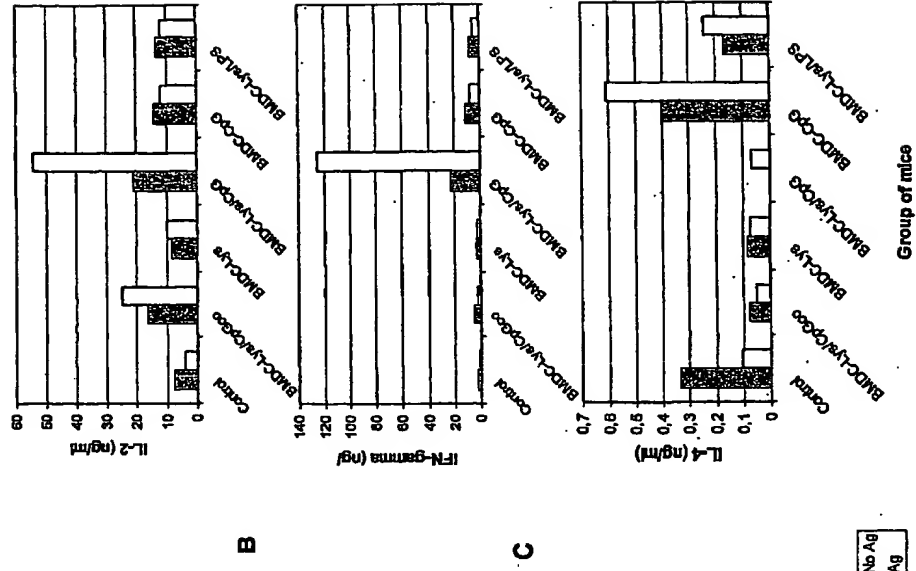


Fig. 4

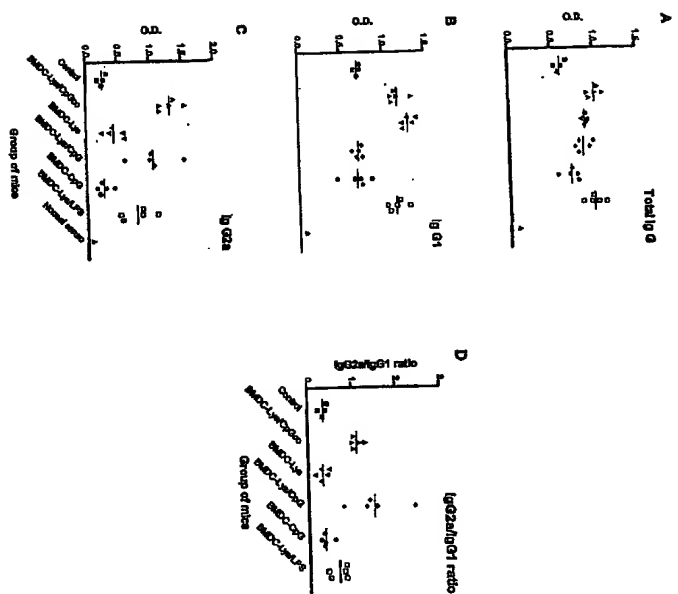


Fig. 5

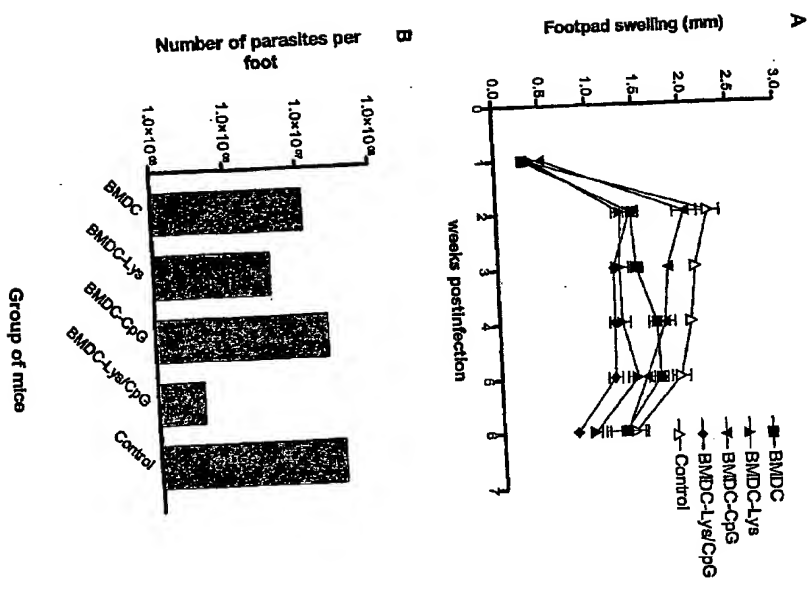


Fig. 6

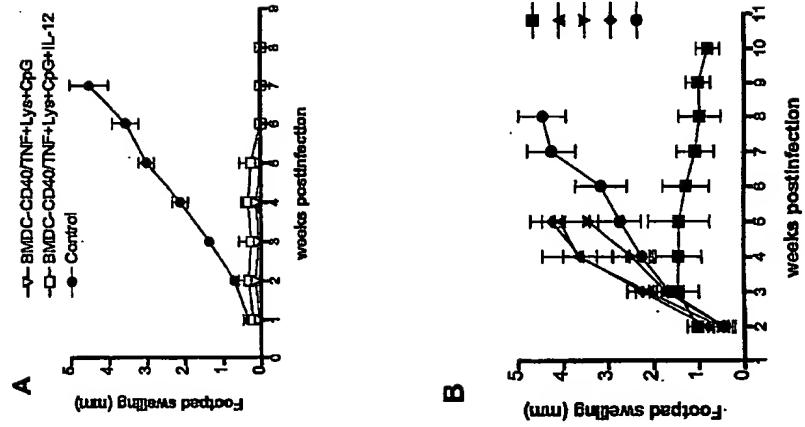
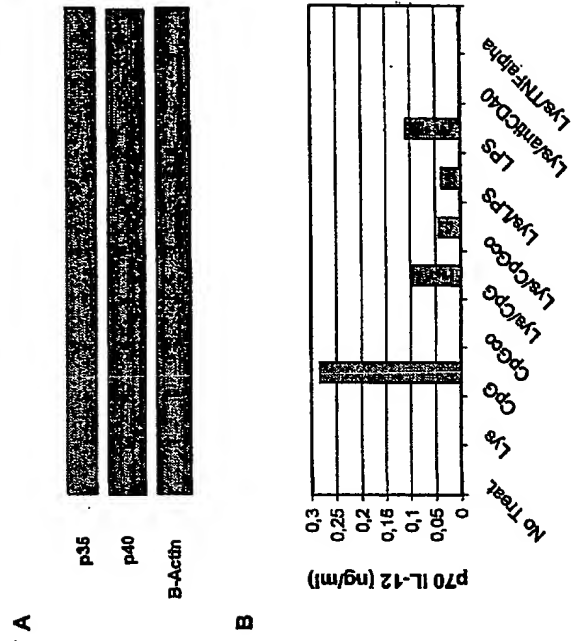


Fig. 7



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C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Referenced to claim No.

 X
 WAGNER H: "BACTERIAL CPB DNA ACTIVATES IMMUNE CELLS TO SIGNAL INFECTIOUS DANGER" ADVANCES IN IMMUNOLOGY, ACADEMIC PRESS INC., NEW YORK, NY, US, vol. 73, 1999, pages 329-368, XP00015569 ISSN: 0065-2776 the whole document, in particular paragraph VI, p. 336-338

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 Further documents are listed in the configuration of item C.

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 C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT
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 X
 JAKOB T ET AL: "ACTIVATION OF CUTANEOUS DENDRITIC CELLS BY CPB-CONTAINING OLIGODEONUCLEOTIDES: A ROLE FOR DENDRITIC CELLS IN THE ALLEGIATION OF TH1 RESPONSES BY IMMUNOSTIMULATORY DNA" JOURNAL OF IMMUNOLOGY, THE WILLIAMS AND WILKINS CO., BALTIMORE, US, 1998, pages 3042-3049, XP002928254 ISSN: 0022-1767 the whole document

X

 WEISBARDT, HEIKE ET AL: "Increased resistance against acute polymicrobial sepsis in mice challenged with immunostimulatory CpG oligodeoxynucleotides is related to an enhanced innate effector cell response" JOURNAL OF IMMUNOLOGY (2000), 165(8), 4537-4543
 XP002262766 the whole document, in particular p. 4537 and "Materials" part

X

SPANASSER ET AL: "Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells" EUROPEAN JOURNAL OF IMMUNOLOGY, WEINHEIM, DE, vol. 28, no. 6, June 1998 (1998-06), pages 2045-2054, XP002131393 ISSN: 0014-2980 the whole document, in particular Fig. 8

X

VABULAS R M ET AL: "CpG-DNA activates in vivo T cell epitope presenting dendritic cells to trigger protective antiviral cytototoxic T cell responses" JOURNAL OF IMMUNOLOGY (BALTIMORE, MD.: 1950) UNITED STATES 1 MAR 2000, vol. 164, no. 5, 2372-2378, XP002262727 ISSN: 0022-1767 the whole document, in particular introduction part

-/-

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INTERNATIONAL SEARCH REPORT		Index	Application No
		PCT/EP	03/05567
Category *		Relevant to claim No.	
Category of document, with indication, where appropriate, of the relevant passages			
X	LIPFORD & B ET AL: "CpG-DNA-mediated transient lymphadenopathy is associated with a state of Th1 predisposition to antigen-driven responses." JOURNAL OF IMMUNOLOGY (BALTIMORE, MD.: 1950) UNITED STATES 1 AUG 2000, vol. 165, no. 3, 1 August 2000 (2000-08-01), pages 1228-1235, XP002252785 ISSN: 0022-1767 the whole document	1-15	
X	WO 02 34887 A (SCHERING CORP) 2 May 2002 (2002-05-02) the whole document, in particular examples and claims	1-15	
P, X	US 6 429 199 B1 (HARTMANN GUNTHER ET AL) 6 August 2002 (2002-08-06) the whole document, in particular columns 2-4, examples and claims	1-15	
P, X	GURSEL MAYDA ET AL: "CpG oligodeoxynucleotides induce human monocytes to mature into functional dendritic cells." EUROPEAN JOURNAL OF IMMUNOLOGY, vol. 32, no. 9, September 2002 (2002-09), pages 2617-2622, XP002252728 ISSN: 0014-2980 the whole document, in particular the abstract	1-15	
A	KRIEG A M ET AL: "CPG MOTIFS IN BACTERIAL DNA TRIGGER DIRECT B-CELL ACTIVATION" NATURE, MACMILLAN JOURNALS LTD. LONDON, GB, vol. 374, 6 April 1995 (1995-04-06), pages 546-549, XP002910391 ISSN: 0028-0836		
A	BERNHARD ET AL: "GENERATION OF IMMUNOSTIMULATORY DENDRITIC CELLS FROM HUMAN CD34+ HEMATOPOIETIC PROGENITOR CELLS OF THE BONE MARROW AND PERIPHERAL BLOOD" CANCER RESEARCH, AMERICAN ASSOCIATION FOR CANCER RESEARCH, BALTIMORE, MD, US, vol. 55, 1 March 1995 (1995-03-01), pages 1099-1104, XP002085385 ISSN: 0008-5472		

Form PCT/ISAR/95 (Publication of national phase) (July 1992)

INTERNATIONAL SEARCH REPORT		Index	Application No
		PCT/EP	03/05567
Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0234887	A 02-05-2002	AU 2893202 A WO 0234887 A2 US 2003003579 A1	06-05-2002 02-05-2002 02-01-2003
US 6429199	B1 06-08-2002	US 6239116 B1 6207646 B1 6194388 B1 2003100527 A1 5242488 A 1238609 A 0948510 A1 2001503267 T 2000052994 A 335397 A 9818810 A1 2003050261 A1 2003026782 A1 2003050263 A1 713040 B2 1912795 A 2194761 A1 1167377 A2 1167378 A2 1167379 A2 0772619 A1 10506265 T 2003144194 A 9602555 A1	29-05-2001 27-03-2001 27-02-2001 29-05-2003 22-05-1998 17-11-1999 13-10-1999 13-03-2001 25-08-2000 24-11-2000 07-05-1998 13-03-2003 06-02-2003 13-03-2003 18-11-1999 16-02-1996 01-02-1996 02-01-2002 02-01-2002 02-01-2002 14-05-1997 23-08-1998 20-05-2003 01-02-1996

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